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Coinhibition of Viral Interferon Induction by Benzo[a]pyrene and Chrysotile Asbestos

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Benzo[a]pyrene (B[a]P) and its noncarcinogenic analog, benzo[e]pyrene (B[e]P), each in combination with chrysotile, were studied for their inhibitory effects on interferon (IFN) induction by influenza virus in rhesus monkey kidney (LLC-MK₂) cell monolayers. B[a]P alone had no adverse effect on IFN induction; however, from 60 to 70% inhibition of IFN production occurred when B[a]P was enzymatically activated by rat liver S9. Chrysotile's inhibitory effect on the IFN process was similar in magnitude to that of activated B[a]P. The combination of activated B[a]P with chrysotile resulted in coinhibition of IFN induction which significantly exceeded ($P < 0.05$) the inhibitory activity of the reagents tested alone or in other combinations. B[e]P alone or with S9 neither affected IFN induction nor was it capable of further enhancing chrysotile's inhibition of IFN synthesis. These findings provide further evidence of enhanced deleterious action by the combination of asbestos and activated B[a]P on a biological defense mechanism and further support the discriminatory power and credibility of the inhibition IFN induction assay for evaluating potential carcinogens. © 1986 Academic Press, Inc.

INTRODUCTION

Although epidemiologic and experimental evidence have shown an association between chronic exposure to asbestos and lung cancer (Wagner *et al.*, 1960; Selikoff *et al.*, 1964; Wagner *et al.*, 1973), studies involving bacterial and cellular mutagenicity (Chamberlain and Tarmy, 1977; Reiss *et al.*, 1982; Reiss *et al.*, 1983), sister chromatid exchanges, and chromosome aberrations (Kaplan *et al.*, 1980; Price-Jones *et al.*, 1980) tend to support the concept that asbestos fibers do not act primarily as genotoxic carcinogens by inducing alterations in DNA (Weisburger and Williams, 1980). Asbestos, though seeming to be weakly carcinogenic, may potentiate or promote the adverse activities of carcinogenic hydrocarbons (Selikoff and Lee, 1978; Lakowicz and Bevan, 1980), i.e., polycyclic aromatic hydrocarbons (PAH). The increased risk of respiratory tract cancer in asbestos workers who smoke cigarettes (Hammond and Selikoff, 1973) may involve synergistic or cocarcinogenic interactions between asbestos and benzo[a]pyrene (B[a]P), a major PAH component in tobacco smoke. Several experimental studies have demonstrated enhanced biologic responses resulting from the combined exposure to B[a]P and asbestos (Reiss *et al.*, 1983; Mossman and Craighead, 1981; Di Paola *et al.*, 1982; Szyba and Lange, 1983; Poole *et al.*, 1983; Chang *et al.*, 1983).

To explore further the biologic responses resulting from exposure to asbestos and B[a]P, the effect of these agents on viral induction of interferon (IFN) was studied. The IFN system, an important component of the host's nonspecific immunologic defense mechanisms, is regarded as a primary protective determinant activated against viral infections. Additionally, IFNs may regulate or modulate various aspects of immune responses and influence the proliferation of both normal and malignant cells as well as different intracellular biochemical activities and products (Taylor-Papadimitriou, 1980). The sensitive nature of the IFN induction process proffers a system based on inhibition of IFN synthesis, for evaluating the inimical biologic potential of mutagens and carcinogens as well as particulates of public health concern (Hahon and Eckert, 1976; Hahon *et al.*, 1979; Hahon *et al.*, 1983; Sonnenfeld *et al.*, 1980; Barnes *et al.*, 1981; Hahon, 1984).

This report describes the *in vitro* conditions under which coinhibition of viral IFN induction occurred when mammalian cell monolayers were exposed to both B[a]P and asbestos.

MATERIALS AND METHODS

Viruses and cell cultures. Virus strains and cell lines used in this study were obtained from the American Type Culture Collection (ATCC), Rockville, Maryland. The Ao/PR/8/34 influenza and parainfluenza (Sendai) viruses, used for interferon induction and assay, respectively, were prepared from embryonated chicken eggs and assayed for virus infectivity by the immunofluorescent cell-counting technique (Hahon *et al.*, 1973). Rhesus monkey kidney (LLC-MK₂) and human Chang conjunctival (clone 1-5c-4) cell lines obtained from ATCC were used for induction and assay of interferon, respectively. Cell lines were propagated in plastic tissue culture flasks (75 cm²) with Eagle's minimum essential medium fortified with 100× Essential Vitamin Mixture (10 ml/liter), 200 μM solution L-glutamine (10 ml/liter) to which was added sodium bicarbonate (2.2 g/liter), and 10% fetal bovine serum. Cells were maintained with the aforementioned medium containing 0.5% fetal bovine serum.

Asbestos and chemicals. Chrysotile asbestos, whose geographic origin was Diablo Range, California and was obtained from the National Institute of Environmental Health Sciences, had a mean length/width of 2.1/0.7 μm. The mineral was made into a stock w/v suspension (1 g/100 ml) in phosphate-buffered saline (PBS), pH 7.1, and sterilized in an autoclave at 20 lb/in² pressure (126°C) for 15 min.

Benzo[a]pyrene (B[a]P) and benzo[e]pyrene (B[e]P), obtained from Sigma Chemical Company, St. Louis, Missouri, were each made into stock solutions by dissolving 10 mg (39.6 μmole) in 10 ml dimethyl sulfoxide (DMSO). Desired test concentrations were prepared in maintenance medium and passed through Millex GS (0.22 μm) filters (Millipore Corp., Bedford, Mass.) to ensure sterility.

Liver homogenate, 9000 g supernatant fraction (S9), was prepared from the livers of male Wistar/Lewis rats (225 g/rat) after induction with Aroclor-1254 (ip, 500 mg/kg body wt) as described by Ames *et al.* (1975). For use in experimental tests, 0.5% suspension of S9 homogenate containing 9.3 mg protein/ml (Lowry *et*

al., 1951) was prepared in maintenance medium and then passed through a Nalge filter unit, 0.45 μm (Nalge Co., Rochester, N.Y., to obtain sterile preparations.

Interferon induction. Duplicate experiments were performed, and the procedure used to study the effect of different combinations of asbestos and chemicals on viral interferon induction was carried out as follows: mixtures consisting of 0.1 or 0.5 mg chrysotile in conjunction with 0.0039 or 0.039 μmole of B[a]P or B[e]P in either 10 ml vol of maintenance medium or 0.5% S9 suspension in maintenance medium were added to 75-cm² plastic flasks containing complete LLC-MK₂ cell monolayers (2×10^7 cells) which were then incubated at 35°C for 24 hr. Residual medium was decanted and 2 ml of influenza virus, which had been inactivated by ultraviolet irradiation for 45 sec at a distance of 76.2 mm and wavelength of 253.7 nm, was added onto cell monolayers that were then incubated at 35°C for 2 hr. The multiplicity of infection (m.o.i.) was approximately 2.0. Inoculum was removed and 10 ml of maintenance medium was added to each flask. After incubation at 35°C for 24 hr, supernatant fluid was decanted and centrifuged at 100,000g for 1 hr and dialyzed against HCl-KCl buffer, pH 2.0, at 4°C for 24 hr. Dialysis was continued against two changes of PBS, pH 7.1, at 4°C for 24 hr. Fluids were passed through Millex filters GV (0.22 μm ; Millipore Corp., Bedford, Mass.) to obtain sterile preparations. Samples were stored at -80°C until they were assayed for interferon activity. Preparations with antiviral activity possessed the biological and physical properties ascribed to viral interferons (Lockhart, 1973). Controls consisting of cell monolayers which were not treated with either asbestos or chemicals were handled exactly as described above.

Interferon assay. An immunofluorescent cell-counting assay of interferon that had been described previously was used to determine the interferon potency of test samples (Hahon *et al.*, 1975). Interferon-treated 1-5c-4 cell monolayers were challenged with 10^4 cell-infecting units of Sendai virus, and infected cells were visualized by direct fluorescent antibody staining. The reciprocal of the interferon dilution that reduced the number of infected cells to 50% of the control served as the measure of interferon activity, i.e., 50% infected cell-depressing dilution (ICDD₅₀). With this assay system, 0.89 interferon unit corresponded to 1.0 unit of National Institutes of Health reference standard Hu IFN β (G-023-902-527).

Statistics Student's *t* and Snedecor's *f* tests were used to determine the statistics of significant levels.

RESULTS

Because cell viability is a prerequisite for IFN induction, the cytotoxic effect of different concentrations of both B[a]P and B[e]P was determined in combination with 0.5 mg chrysotile. LLC-MK₂ monolayers of nondividing cells (2×10^7) were incubated at 35°C for 24 hr with various amounts of chemicals and cell viability was determined by the trypan blue dye exclusion procedure. Under the specified conditions, B[e]P appeared more cytotoxic than B[a]P. Cells were able to tolerate at least a tenfold higher quantity of B[a]P (0.039 μmole) than B[e]P (0.0039 μmole) without appreciable loss of viability (<0.5%). These and lesser amounts of chemicals were used in succeeding experiments.

Different concentrations of B[a]P with and without rat liver S9 and in combination with chrysotile were tested for their effect on viral IFN induction. Results (Table 1) show that B[a]P alone was not detrimental to IFN induction; however, when B[a]P was activated enzymatically by S9, from 60 to 70% inhibition of IFN induction occurred depending on the amount of chemical tested. The inhibitory activity of chrysotile on the process, with or without S9, was comparable to that of activated B[a]P. The combination of B[a]P with S9 and chrysotile resulted in coinhibition of IFN induction. With this mixture, inhibitory activity was significantly greater ($P < 0.05$) than with any of the reagents tested either alone or in other combinations.

Viral IFN induction experiments, similar to those performed with B[a]P and chrysotile, were carried out with the noncarcinogenic analog, B[e]P. Results (Table 2) show that the analog with or without the presence of microsomal enzymes (S9) had no appreciable adverse effect on IFN induction. Chrysotile markedly depressed IFN production but this inhibitory activity was not enhanced by combinations of B[e]P or S9 indicating the absence of any additive adverse effect by these reagents.

TABLE 1
COINHIBITION BY BENZO[a]PYRENE AND CHRYSOTILE ON INTERFERON INDUCTION BY INFLUENZA VIRUS

B[a]P (μ mole)	Chrysotile (mg)	S9 ^a (0.5%)	IFN ^b (ICDD ₅₀)	IFN inhibition ^c (% $\pm$ SEM)
0.039	—	—	3220	1.0 $\pm$ 0.5
0.039	—	+	970	70.2 $\pm$ 2.3
0.039	0.5	—	920	71.7 $\pm$ 0.5
0.039	0.5	+	390	88.0 $\pm$ 2.7 ^d
—	0.5	—	960	70.5 $\pm$ 1.2
—	0.5	+	920	71.7 $\pm$ 0.5
—	—	+	3200	1.6 $\pm$ 1.5
— (Control)	—	—	3250	0.0
0.0039	—	—	1650	0.0 $\pm$ 0.0
0.0039	—	+	620	61.3 $\pm$ 0.8
0.0039	0.1	—	600	62.5 $\pm$ 0.3
0.0039	0.1	+	210	86.9 $\pm$ 0.9 ^d
—	0.1	—	560	65.0 $\pm$ 3.7
—	0.1	+	550	65.7 $\pm$ 3.4
—	—	+	1610	0.0 $\pm$ 0.0
— (Control)	—	—	1600	0.0

^a Rat liver homogenate 9000 g supernatant fraction.

^b Mean reciprocal value of two determinations of 50% infected cell-depressing dilution/10 ml medium from LLC-MK₂ monolayers (2×10^7 cells).

^c Reciprocal IFN ICDD₅₀ — 1.0×100 .
Reciprocal control IFN ICDD₅₀

^d Student's *t* significance level ($P < 0.05$) relative to B[a]P with S9 and B[a]P without S9 plus chrysotile as well as chrysotile with and without S9.

TABLE 2
EFFECTS OF ANALOG BENZO[e]PYRENE AND CHRYSOTILE ON INTERFERON INDUCTION BY
INFLUENZA VIRUS

B[e]P (μ mole)	Chrysotile (mg)	S9 ^a (0.5%)	IFN ^b (ICDD ₅₀)	IFN inhibition ^c (% $\pm$ SEM)
0.0039	—	—	1100	0.0 $\pm$ 3.6
0.0039	—	+	970	9.4 $\pm$ 1.5
0.0039	0.5	—	210	80.4 $\pm$ 8.0 ^d
0.0039	0.5	+	260	75.8 $\pm$ 5.2 ^d
—	0.5	—	240	77.6 $\pm$ 2.0 ^d
—	0.5	+	240	77.6 $\pm$ 9.2 ^d
—	—	+	1040	2.9 $\pm$ 4.6
— (Control)	—	—	1070	0.0
0.0039	—	—	1360	0.0 $\pm$ 1.9
0.0039	—	+	1270	6.0 $\pm$ 1.8
0.0039	0.1	—	490	63.8 $\pm$ 0.7 ^d
0.0039	0.1	+	550	59.3 $\pm$ 11.1 ^d
—	0.1	—	600	55.6 $\pm$ 1.8 ^d
—	0.1	+	530	60.8 $\pm$ 2.2 ^d
—	—	+	1350	0.0 $\pm$ 0.9
— (Control)	—	—	1350	0.0

^a Rat liver homogenate 9000 g supernatant fraction.

^b Mean reciprocal value of two determinations of 50% infected cell-depressing dilution/10 ml medium from LLC-MK₂ monolayers (2×10^7 cells).

^c Reciprocal IFN ICDD₅₀ $\sim 1.0 \times 100$.
Reciprocal control IFN ICDD₅₀

^d No difference among values in each set at Snedecor's *f* significance level ($P < 0.05$).

DISCUSSION

The findings reported herein demonstrate that B[a]P in combination with a commonly used form of asbestos (chrysotile) resulted in coinhibition of viral IFN induction that significantly exceeded ($P < 0.05$) the inhibitory activity of each reagent tested alone. B[a]P enzymatically activated by rat liver S9 to carcinogenic metabolites was a requisite to this phenomenon because B[a]P alone displayed no appreciable detrimental activity. Using second or third passage mouse embryo fibroblast cell cultures, DeLor and Sonnenfeld (1984) reported that B[a]P without S9 supplementation could inhibit interferon induction. Conceivably, this could be related to the cell strain per se or its early passage history. It was previously shown with the established cell line (LLC-MK₂) used herein that the activation-dependent carcinogen, 2-aminoanthracene, and other azo dyes required the presence of S9 (Hahon, 1984) to demonstrate depression of IFN synthesis (Hahon and Eckert, 1976), but different forms of asbestos did not. That asbestos alone can readily inhibit IFN induction contrasts markedly to difficulties experienced in demonstrating its possible mutagenic or carcinogenic potential in other test systems (Chamberlain and Tarmy, 1977; Reiss *et al.*, 1982; Kaplan *et al.*, 1980; Mossman *et al.*, 1983). B[e]P, a noncarcinogenic analog of B[a]P, alone or

with S9, neither affected IFN induction nor enhanced the inhibitory action of chrysotile on the IFN process. The discriminatory power and credibility of the inhibition IFN induction assay for evaluating potential carcinogenic agents is further supported by these findings.

The mechanism by which coinhibition of viral IFN induction occurs with enzymatically activated B[a]P in combination with asbestos fibers is not clear. The transport, uptake, and retention into cells of B[a]P may be facilitated by the adsorptive power of asbestos or the latter's alteration of plasma membrane permeability (Lakowicz and Hylden, 1978; Lakowicz *et al.*, 1978; Chang *et al.*, 1983; Mossman *et al.*, 1983). It is also possible, as demonstrated in bacterial mutagenicity tests, that the carcinogen may be more accessible to microsomal enzymes when it is adsorbed onto asbestos fibers, thereby resulting in increased efficiency of metabolic activation (Szyba and Lang, 1983). That the demonstration of coinhibition by B[a]P and chrysotile may be a purely additive effect with each reagent occupying different cellular target sites is another consideration. Nonetheless, these may be only inchoate interactions as other activities most probably are involved in carcinogenesis and in the described inhibition of interferon synthesis. Although alteration of the IFN cellular defense mechanism may play a role in the cocarcinogenic potential of B[a]P in combination with asbestos, a paucity of information at the host, cellular, and molecular levels limits our understanding of the complex mechanisms involved and the status of the IFN system in this phenomenon.

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